(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: 0 196 761
A2

(12) # EUROPEAN PATENT APPLICATION

(21) Application number: 86301196.1

(22) Date of filing: 20.02.86

(51) Int. Cl.⁴: A 61 L 2/04

(30) Priority: 21.02.85 JP 33335/85

(43) Date of publication of application:
08.10.86 Bulletin 86/41

(84) Designated Contracting States:
BE CH DE FR GB LI NL SE

(71) Applicant: THE GREEN CROSS CORPORATION
15-1, Imabashi-1-chome Higashi-ku Osaka-shi
Osaka 541(JP)

(72) Inventor: Hirao, Yutaka
14-1-606, Terauchi-2-chome
Toyonaka-shi(JP)

(72) Inventor: Uriyu, Katuhiro
601-32, Nakatsumichi-3-chome
Daifuku Sakurai-shi(JP)

(72) Inventor: Uemura, Yahiro
Mezon Hirakata 215 5-18, Mitsuyacho
Hirakata-shi(JP)

(74) Representative: Harrison, David Christopher et al,
MEWBURN ELLIS & CO 2/3 Cursitor Street
London EC4A 1BQ(GB)

(54) **Method of virus-inactivating heat treatment of gamma-globulin.**

(57) A gramma-globulin aqueous solution can be subjected to sterile heat-treatment without losing its activity when the treatment is conducted in the presence of at least one stabilizer which is a monosaccharide, disaccharide or sugar-alcohol added to the solution. The stabilizer serves to decrease the harmful polymer or anticomplement activity of the gamma-globulin before the treatment but to maintain the titer of a wide variety of antibodies to various viruses and bacteria, while the infectivity of viruses possibly contained is completely removed.

An additional stabilizer, which is a neutral amino, a neutral inorganic salt, an organic carboxylic acid salt or a surface active agent, assists the abovementioned stabilizer when used together with it.

EP 0 196 761 A2

0196761

METHOD OF VIRUS-INACTIVATING HEAT TREATMENT OF γ-GLOBULIN

This invention relates to a method of the heat treatment of an aqueous solution containing γ-globulin. More particularly, it relates to a method of a stable heat treatment of γ-globulin wherein a selected stabilizer is added to an aqueous solution of γ-globulin and no increase of the dimer or polymer of γ-globulin nor increase in anticomplement activity is observed after low temperature pasteurization, i.e. a treatment conducted at 60°C for 10 hours.

Gamma globulin preparations containing, among immunoglobulins of a plasma protein component, particularly IgG as the principal ingredient have been widely used in the prevention and the treatment of various infectious diseases. However, they are not subjected to a heat-sterilization for the reason that they have a poor heat stability and that they contain a wide variety of antibodies to various viruses and bacteria, of which activity is liable to be lost.

However, when γ-globulin is prepared from the fraction of plasma protein, the possibility of contamination with viruses such as hepatitis viruses cannot be negated completely. Accordingly, it is important that γ-glubulin preparations are subjected beforehand to a heat treatment at 60°C for 10 hours, which treatment has been widely recognized in the blood component preparation

technology as a method for inactivating contaminating viruses.

However, when the treatment is conducted in a conventional aqueous solution such as physiological saline solution, the solution becomes turbid in a short time, most of the activity being lost and the protein molecules being denatured.

After extensive studies, the present inventors have found that the thermal stability of γ-globulin against heat treatment is markedly improved when at last one member (hereinafter referred to generically as a principal stabilizer) selected from the group consisting of a monosaccharide, a disaccharide, and a sugaralcohol is added to an aqueous solution containing γ-globulin prior to or at the time of the heat treatment of the solution for inactivating hepatitis viruses and that the thermal stability of γ-globulin is further enhanced when at least one member (hereinafter referred to generically as an auxiliary stabilizer) selected from the group consisting of a neutral amino acid, a neutral inorganic acid salt, a surface active agent, and an organic carboxylic acid salt is added to the solution in addition to the said principal stabilizer. This invention has been accomplished on the basis of above findings.

Further, the heat treatment according to the method of this invention make it possible to dissociate the dimer or polymer of γ-globulin contained in the aqueous γ-globulin solution into its monomer.

The aqueous solution containing γ-globulin to be heat-treated according to this invention may be an aqueous γ-globulin solution at any stage of purification ranging from an unpurified aqueous solution containing γ-globulin to a purified aqueous solution. However, an aqueous solution at a partially purified or purified stage is advantageously subjected to the heat treatment. The aqueous solution preferably contains 0.1 to 30% (w/v) of protein (γ-globulin). The pH of the aqueous solution is preferably generally 4.5 to 10, and more preferably adjusted to pH 6 to 8 with a suitable buffer solution.

As to the principal stabilizers added to the aqueous solution containing γ-globulin, preferred examples of a mono-saccharide include glucose, mannose, galactose, and fructose, those of a disaccharide include sucrose, maltose, and lactose, and those of a sugaralcohol include mannitol, sorbitol and xylitol, but they are not limited to these examples. The amount of the principal stabilizer to be added is preferably 10 to 100 g, more preferably 40 to 100 g per 100 ml of aqueous γ-globulin solution.

Among the auxiliary stabilizers used in this invention, the neutral inorganic acid salts include, for example, the halide of alkali metals or alkaline earth metals such as sodium chloride, potassium chloride, and magnesium chloride. Their amount to be added is preferably 0.1 to 10 g per 100 ml of aqueous γ-globulin solution.

Examples of the neutral amino acids (usually, monoaminomonocarboxylic acids) include glycine, alanine,

valine, leucine, and isoleucine. Their amount to be added is preferably 1 to 20 g per 100 ml of aqueous γ-globulin solution.

The organic carboxylic acid referred to in this invention is a compound comprising a hydrocarbon residue and a carboxyl substitute attached thereto. The hydrocarbon residue may be either saturated or unsaturated, and either chain-like (straight chain or branched chain) or cyclic. Examples of the hydrocarbon residue include an alkyl group and an aryl group (such as a phenyl group). The number of carboxyl groups in said organic carboxylic acid may be plural, but is preferably one or two. Further, said organic carboxylic acid may have a hydroxyl group. The organic acid has preferably about 3 to about 15 carbon atoms.

The kind of the salts of the organic carboxylic acids is not particularly restricted so long as it is physiologically acceptable. Preferred examples thereof include alkali metal salts such as sodium salts and potassium salts and alkaline earth metal salts such as calcium salts. Particularly preferable are sodium salts and potassium salts. The specific examples of the organic acid salts include particularly alkali metal salts (sodium or potassium salt) of propanoic, butanoic, pentanoic, caprylic, caproic, malonic, succinic, glutaric, adipic, citric and mandelic acid. The amount of the organic carboxylic acid salt to be added is 1 to 30 g per 100 ml of the aqueous γ-globulin solution.

Examples of the surface active agents usable in

this invention include nonionic surface active agents such as alkylphenyl-polyoxyethylene having a molecular weight of 500 to 1,000 [for example, Triton (a registered trade mark) and Nonidet (a registered trade mark)], anionic surface active agents such as bile acid salts, for example sodium taurocholate, cationic surface active agents such as benzalkonium chloride, and polyhydric alcohols having surface activity such as a high molecular weight copolymer of propylene oxide having a molecular weight of 2,000 to 12,000 [for example, Pluronic (a registered trade mark) F68]. Their amount to be added is preferably about 0.002 to about 0.05 g per 100 ml of the aqueous γ-globulin solution.

The heat treatment should be conducted at a sufficient temperature and for a sufficient time for inactivating contaminating viruses only. For example, it is conducted at 50 to 70°C, preferably at about 60°C, for 5 to 20 hours, preferably for 10 hours.

In order to examine the effect of the heat treatment according to this invention, the effect of heating in the presence of a principal stabilizer and that in the absence of the principal stabilizer were tested in the following manner on the infectivity of various viruses whose possible presence in γ-globulin preparations is apprehended. Thus, smallpox viruses, parotitis viruses, measles viruses, vesicular viruses, chikungunya viruses, polioviruses, coxsackie viruses, or echoviruses were added to a γ-globulin solution specimen, the resulting mixture

was heat-treated at 60°C for 10 hours, and the remaining infectivity of the viruses was determined with the lapse of time. The infectivity was found to have had vanished completely after 10 hours irrespective of the presence or the absence of the stabilizer. The result suggests that other viruses than those used above will lose their infectivity when heat-treated according to this invention.

After the above-mentioned heat treatment in the presence of the principal stabilizer according to this invention, the resulting product is examined for its appearance and properties and further subjected to the quantitative determination of dimer or polymer of γ-globulin, the determination of anticomplement activity, the determination of measles antibody titer, and the acute toxicity test. The results obtained reveal as disclosed in the Example below, the decrease of the dimer or polymer and anticomplement activity of γ-globulin, but the remaining of the antibody titer, showing that it gives a γ-globulin preparation exhibiting an extremely high safety and a high effectiveness in medical treatment.

The product thus obtained is in a liquid state, and is dispensed, as it is when a highly purified γ-globulin has been used as the starting material and after treated according to a known method of purification followed, as required, by dialysis or sterile filtration when it has been derived from a crude product, so as to contain 50 to 10,000 mg of γ-globulin depending on package units. The method of its storage is not particularly

restricted so long as a high temperature is avoided. However, it is particularly preferably stored at a temperature not higher than 30°C or, as desired, may be made into a lyophilized preparation.

The γ-globulin thus treated is then administered as it is or after a preparation treatment known per se, for example after being diluted by or dissolved in or dialyzed against distilled water which may be for injection use. The usual dosage is 2,500 to 5,000 mg/kg body weight in terms of γ-globulin per one time for adults and 100 to 150 mg/kg body weight in terms of γ-globulin per one time for infants.

The present invention is further explained by the following Examples, but it is not limited thereto.

In the Examples, in terms of the appearance, absorbance, O.D. 600 nm, was determined since turbidity becomes a problem.

The quantity of the dimer or the polymer was determined by means of high performance liquid chromatography.

The anticomplement activity was determined according to the method of Kabatt and Meyer [Experimental Immunochemistry, 225 (1961)] and the method of Nishioka and Okada [Men'eki no Seikagaku (Biochemistry of immunity) 103, (1971); published by Kyoritsu Shuppan Co.]. Namely, a specimen was added to 100 units of complement and the number of units remaining in the resulting mixture was determined. The anticomplement activity were expressed

in terms of the decreased units.

The measles antibody titer was determined by hemagglutination inhibition test and expressed in terms of international units (IU/150 mg).

Example 1

Experiments were made to confirm the stabilizing effect according to this invention. The experiments were conducted with samples prepared by adjusting a solution of a γ-globulin containing about 30% of polymer to a concentration of 5%. After the addition of various principal stabilizers (the amount added being indicated in the Table 1), the sample was heat-treated at 60°C for 10 hours and then examined for the turbidity (O.D. 600 nm) of the solution, the quantity of polymer and the anti-complement activity. The results obtained revealed that the stability of γ-globulin in heating was improved by the addition of stabilizer (Table 1).

Further, the decrease of amount of polymer, particularly dimer, was confirmed.

Table 1

Stabilizer	Amount added *1	O.D. 600 nm	Polymer (%)		Anticomplement activity (unit)
			Dimer	Polymer	
Control (before heating)	-	0.024	33	2	54
None (for comparison)	-	Turbid	- *2	-	-
Glucose	50	0.010	15	2	38
Sucrose	50	0.012	13	2	36
Manitol	20	0.017	17	2	42

Note: *1: Amount (g) per 100 ml of 5% (w/v) γ-globulin solution

*2: So much as cannot be determined.

Example 2

Glucose was added in various concentrations to a γ-globulin solution containing about 20% (w/v) of polymer and the concentration of γ-globulin in the resulting mixture was adjusted to 5% (w/v). The solution thus obtained was heat-treated at 60°C, and the value of O.D. 600 nm, the quantity of polymer, the anticomplement activity, and the measles antibody titer were determined with the lapse of time.

The system containing no glucose became turbid within one hour, showing the occurrence of denaturation. The systems containing added glucose showed increasing stability of γ-globulin with increasing amount of glucose added. The system to which 100 g of glucose had been added did not become turbid and showed no decrease in the measles antibody titer even after heated at 60°C for 10 hours. Further, the content of dimer decreased down to only 10% and the anticomplement activity also decreased down to 19 units (Table 2).

Table 2 Heat treatment at 60°C for 10 hours

Amount of glucose added (g)	O.D. 600 nm	Polymer (%)		Anticomplement activity (unit)	Measles antibody titer (IU)
		Dimer	Polymer		
Control (before heating)	0.024	22	3	54	42
None	Turbid	- *1	-	-	-
5	Turbid	-	-	-	-
25	0.040	15	30	>50	<10.5
50	0.010	13	3	36	21
75	0.004	12	2	28	40
100	0.004	10	2	19	40

Note: *1: So much as cannot be determined.

Example 3

In addition to the principal stabilizer, glucose, one or two auxiliary stabilizers selected from a neutral amino acid (glycine), a neutral salt (sodium chloride), an organic carboxylic acid salt (sodium citrate), and a surface active agent (Pluronic® F68), were added to a γ-globulin solution and the stability of γ-globulin in the resulting solution in heat treatment at 60°C for 10 hrs was examined. The test was conducted with a γ-globulin solution same as in Example 1 but containing about 15% (w/v) of polymer.

Heat treatment at 60°C for 10 hours was conducted for each of the systems to which 75 g of glucose was added in common per 100 ml of γ-globulin solution and 5.8% (w/v) of sodium chloride, 5% (w/v) of glycine, 10% (w/v) of sodium citrate, 0.01% (w/v) of Pluronic® F68, or a combination of two auxiliary stabilizers, 5.8% (w/v) of sodium chloride and 0.01% (w/v) of Pluronic® F68, was added. The results obtained are shown in Table 3. The results reveal that the content of polymer and the anticomplement activity can be further decreased by the addition of the auxiliary stabilizers.

Table 3

Auxiliary stabiliser (Referred to in Example 4)		Amount added (g)	O.D. 600 nm	Polymer (%)		Anti-complement activity (unit)	Measles antibody titer (IU)
				Dimer	Polymer		
Control (before heating)		-	0.004	15	2	44	42
None	(A)	-	0.004	8	1	28	40
Sodium chloride	(B)	5.8	0.004	5	1	18	42
Glycine	(C)	5	0.006	8	2	25	45
Sodium citrate	(D)	10	0.004	8	1	24	38
Pluronic® F68	(E)	0.01	0.004	6	1	13	40
Sodium chloride Pluronic® F68	(F)	5.8 0.01	0.004	6	1	12	41

0196761

1 Example 4

Acute toxicity tests were conducted by way of a safety test.

Samples A, B, C, D, E and F which had been heat-treated at 60°C for 10 hours in Example 3 were dialyzed thoroughly against sterile physiological saline, and then administered respectively to mice in groups of five through the tail vein in a total amount of 0.5 ml and 1.0 ml per one animal. No abnormality was found after 7 days of observation.

CLAIMS:-

1. A method of a virus-inactivating heat treatment of an aqueous γ-globulin solution, comprising adding to the aqueous solution an amount sufficient for stabilizing γ-globulin therein of at least one stabilizer selected from the group consisting of a monosaccharide, a disaccharide and a sugaralcohol, and heating the aqueous solution at a temperature of 50° to 70°C for a period sufficient substantially to free the aqueous γ-globulin solution from infectivity of a virus.

2. The method of Claim 1, wherein the aqueous solution contains 0.1 to 30% (w/v) of γ-globulin in terms of protein.

3. The method of Claim 1, wherein the aqueous solution has a pH of 4.5 to 10.

4. The method of Claim 1 or 2, wherein the amount of the stabilizer is 10 to 100 g per 100 ml of the solution.

5. The method of Claim 4, wherein the amount of the stabilizer is 40 to 100 g per 100 ml of the solution.

6. The method of any preceding Claim, wherein in addition to the stabilizer, an amount effective for decreasing anti-complement activity as compared with the use of the stabilizer alone, of at least one auxiliary stabilizer selected from the group consisting of a neutral amino acid, a neutral inorganic acid salt, an organic carboxylic acid having 3 to 10 carbon atoms and a surface active agent is added to the aqueous solution.

7. The method of Claim 6, wherein the amount of the neutral amino acid is 1-20 g per 100 ml of the solution.

8. The method of Claim 6, wherein the amount of organic carboxylic acid is 1-30 g per 100 ml of the solution.

9. The method of Claim 6, wherein the amount of the inorganic acid salt is 0.1 to 10 g per 100 ml of the solution.

10. The method of Claim 6, wherein the amount of the surface active agent is 0.002 to 0.05 g per 100 ml of the solution.